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PRODUCTION OF PROTEOLYTIC ENZYME FROM A LOCAL STRAIN OF *BACILLUS SUBTILIS*

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ABSTRACT

From 990 bacterial isolates, a *Bacillus subtilis* strain isolated from soil was selected based on its protease-producing efficiency of 62.50 (Oshima and Church value and its ability to produce clear zones on casein agar. On soybean medium, optimum pH for protease formation is 6.0 to 7.5. Trace elements, zinc and calcium, are stimulatory to protease production. A yield of 1.5 per cent protease with a proteolytic value of 1,660 was obtained from Le Mense medium containing 10 per cent soybeans.

The Philippines abounds with readily available supply of protein-rich materials such as soybeans, copra meal, and rice bran. These substances can be used as substrates for the production of enzymes, like proteases, which are of great commercial value.

Proteolytic enzymes have varied uses in industry. It is an important component of detergents for use in laundering

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separate test tubes. The digestion was carried on for 1 hour at 37 to 40°C. The appearance of cloudiness or precipitate after adding 0.5 ml of a mixture of saturated $MgSO_4$ solution and concentrated HNO_3 to the digestion tubes indicates incomplete digestion. The density of cloudiness or precipitation is dependent upon the amount of undigested casein. The clear tube next to the one showing opacity was taken as the tube containing the minimum amount of enzyme which digested completely the casein in the 5 ml of a 0.5 per cent solution.

To express the proteolytic power, the following formula devised by Oshima and Church was adopted: If 0.025 g or ml of the original enzymic substance digests completely 5 ml of 0.5 per cent casein solution (0.025 g casein) in 1 hour at 40°C, then the proteolytic value (PV) of this substance is 100.

The isolate which produced the highest PV was selected and used in all succeediing studies on its characterization and cultural requirements for protease production. The rest of the isolates, weak producers of protease, were discarded.

Studies on cultural requirements for protease production by the selected isolate.—All experiments described hereunder were carried in 250-ml Erlenmeyer flasks, each containing 50 ml of Le Mense medium and 10 per cent ground soybeans. Sterilization was done by autoclaving the medium for 20 minutes at 15 psi. The inoculant consisted of an actively growing 48-hour-old culture of the bacterium in tryptone-glucose-yeast agar medium. Aeration was supplied by shaking in a reciprocal shaker with a speed of 63 cycles per minute for 4 days. The culture filtrate was obtained by passing the soybean cultures repeatedly through filter paper until it was clear. The PV of the culture filtrate was determined by following the procedure of Oshima and Church.[7]

To select the best concentration of soybeans for enzyme production, 5, 10, 15, and 20 per cent ground soybeans were incorporated in separate media.

To determine the best age of inoculum for the production of protease, various ages of the isolate ranging from 1 to 7 days with an interval of 1 day was used to inoculate the medium.

To know the effects of incubation period on production of protease, 5-ml samples of the culture medium were taken daily for 8 days and the PV of the culture filtrate was determined.

The influence of pH on growth and protease production was conducted by growing the selected isolate in soybean medium adjusted with the aid of a Beckman Zeromatic pH meter to pH levels ranging from 4.0 to 10.0 at intervals of 0.5.

The trace elements requirements for growth and protease production by the soil bacterium was determined by adding the elements derived from Hutner's formula* used by Burlew [10] for algal cultures. Each of the salt was added in 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 ml portions per 100 ml of the medium. A medium free of trace elements was used as control.

Precipitation of the enzyme.—The active enzyme was precipitated by following the method of Hoogerheide.[4] The protease from the clear culture filtrate was precipitated by adding 2 volumes of ethyl alcohol. The precipitate was separated from the supernatant liquid by filtration and air dried in vacuum.

Characterization and identification of the protease-producing bacterium.—The cellular, cultural and physiological characteristics of the selected soil bacterium were studied by following the procedures of Jacobs and Gerstein, [11] Clark and Halvorson,[5] Breed *et al,* [12] and the bacteriological methods adopted by the Society of American Bacteriologists.[13]

To check the identity of the isolate, similar tests were conducted simultaneously using an identified *Bacillus subtilis* strain.

RESULTS AND DISCUSSION

Selection of the promising protease-producing bacterium.—Further testing of the 860 bacterial isolates selected from the preliminary screening showed that 353 were protease pro-

* Microelement solutions:

Salt	*Stock solution (g/l)*
$CaCl_2$	8.34
H_3BO_3	11.42
$FeSO_4.7H_2O$	4.98
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
H_2MoO_4	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

ducers and 507 were nonprotease-producers. Of the protease producers only 17 were able to produce a PV of 50.0 to 83.33 (Oshima and Church value). After repeated testing of the potency of the 17 selected isolates only three retained the original PV of 62.50. The PV's of the rest of the isolates were very unstable as shown by the decrease in enzymic activity after several determinations.

Hoogerheide[6] reported that the *Bacillus subtilis* strains tend to degenerate and are subject to considerable variation beyond the control of the investigator. This may explain the inconsistency of the PV of some of the isolates. For this reason, the stock cultures of the selected isolates with high potency were preserved immediately by paraffin seal or by lyophilization to minimize if not totally prevent any change in activity.

On the basis of repeated trials on the potency of the protease-producing bacteria, isolate No. B–832 was selected as the most efficient since the PV of 62.50 remained constant after several determinations. All subsequent studies were made on this isolate.

Selection of the best concentration of soybeans for enzyme production.—Table 1 illustrates that when the bacterium was cultured in 10 and 15 per cent soybeans in Le Mense medium, the maximum amount of enzyme, PV 62.50, was produced.

TABLE 1.—*Proteolytic values of enzyme produced from different concentrations of soybeans.*

Concentration of soybeans (Per cent)	Average proteolytic value (PV)
5	12.50
10	62.50
15	62.50
20	—

In 5 per cent soybean medium, very low activity was obtained, PV 12.50. Using 20 per cent soybeans, the organism failed to produce the enzyme because of the thick consistency of the medium. The 10 per cent concentration of soybeans was found to be the best and was therefore used in succeeding studies.

The isolate grows submerged and on the surface of the medium forming a more or less wrinkled pellicle (Plate 1). Hoogerheide [4] cited the importance of a good heavy pellicle for amylase production. It is likewise important to have a pellicle formation during protease production, since it was observed that the PV of the enzyme was improved when the surface growth was present. It was also noted that protease formation decreased when shaking exceeded 63 cycles per minute. Davies [14] reported that some enzymes are very susceptible to surface denaturation when the area of the gas-water interface is greatly increased by vigorous gassing or shaking. This is not an oxidation effect.

Influence of age of inoculum on protease production.—Good growth of the bacterium as shown by a heavy wrinkled pellicular formation was produced in all batches inoculated with the isolate grown at various ages. In Table 2, the PV of 62.50 was highest in the medium which was inoculated with 48- to

TABLE 2.—*Relation of age of inoculum to protease formation by the local strain of Bacillus subtilis.*

Age of inoculum (Days)	Average proteolytic value (PV)
1	53.19
2	62.50
3	62.50
4	60.97
5	60.97
6	57.34
7	38.46

72-hour-old bacterium. The activity was lowest, PV 38.46 in the batch of the 1-week-old inoculum. This demonstrates that the isolate, being a spore-forming organism favors the production of enzyme during the sporulating stage.

Kay [15] stated that spore formation by *Bacillus subtilis* is considered to start at the end of the period of exponential growth, during which, growth rate is maximal. This explains

why at this stage the 48- to 72-hour-old cultures produced the maximum amount of protease whereas older cultures which are probably in the stationary phase tended to produce less enzyme.

Relation of incubation period to protease formation.—Table 3 illustrates that the amount of protease produced by the isolate during the first day was PV 11.45. On the second day to the third day, the PV started to increase from 45.45 to 49.03 to 62.50 and remained constant at this level from the fourth

TABLE 3.—*Influence of length of incubation period to protease formation by the soil strain of B. subtilis.*

Period of incubation (Days)	Average proteolytic value (PV)
1	11.45
2	45.45
3	49.03
4	62.50
5	62.50
6	62.50
7	60.50
8	50.00

day to the sixth day. On the seventh day, the PV of the culture filtrate decreased to 60.50 and as the organism became older, the amount of available protease was correspondingly decreased to PV 50.00. This may be attributed to exhaustion of nutrients at the stationary phase of growth of the bacterium. This pattern of protease formation is substantiated in the statement of Warren [16] that toward the end of exponential growth, proteolytic activity increased and reached the maximum soon after growth ceased.

Effect of pH on protease production.—The results shown in Table 4 indicate that the soil bacterium can tolerate a wide range of initial pH. The amount of available protease, PV 83.33 was maximum at pH 6.0 to 7.5 with a slight decrease at pH 4.5 to 5.0 and 8.0 to 8.5. Only the extremely acidic and alkaline medium yielded a low PV of 35.71 at pH 4.0 and 6.25 at pH 10.0.

The ability of the organism to produce proteolytic enzyme over a wide range of pH extending from 4.0 to 9.0 is an

TABLE 4.—*Comparative proteolytic values of protease produced by the soil isolate at various pH levels.*

Initial pH of medium	Average proteolytic value (PV)
4.0	35.71
4.5	62.50
5.0	62.50
5.5	62.50
6.0	83.33
6.5	83.33
7.0	83.33
7.5	83.33
8.0	78.12
8.5	69.44
9.0	59.52
9.5	50.00
10.0	6.25

extremely valuable characteristic from the point of view of commercial application. Since protease can be produced by the bacterium at alkaline levels, the enzyme may be useful as an additive in detergents. Maxatase, a proteolytic enzyme additive in laundry compounds, is produced from a special strain of a spore-forming *Bacillus* by the Royal Netherland Fermentation Industries Ltd.

Trace elements influencing protease production.—Table 5 shows that protease was produced in all batches of soybean medium containing various trace elements except $FeSO_4$ which had an inhibiting effect on the production of enzyme. The

TABLE 5.—*Effect of different trace elements on protease formation in soybean medium.*

Trace elements	Amount of trace element solutions per 100 ml of Le Mense medium (ml)	Average proteolytic value (PV)
	5	62.50
	10	83.33
	15	83.33
	20	83.33
$CaCl_2$	25	83.33
	30	75.72
	35	75.72
	40	75.72
	45	62.50
	50	62.50

Trace elements	Amount of trace element solutions per 100 ml of Le Mense medium (ml)	Average proteolytic value (PV)
H_3BO_3	1	60.97
	2	59.52
	3	64.27
	4	61.73
	5	58.52
$FeSO_4.7H_2O$	1	62.50
	2	61.73
	3	27.77
	4	6.25
	5	—
$ZnSO_4.7H_2O$	5	78.12
	10	81.98
	15	81.98
	20	119.30
	25	119.30
	30	108.69
	35	108.69
	40	35.71
	45	35.71
	50	35.71
$MnCl_4.4H_2O$	5	62.50
	10	62.50
	15	62.50
	20	75.72
	25	58.13
	30	55.66
Silico-molybdenum complex	5	62.50
	10	80.64
	20	80.64
	15	73.52
	25	62.50
	30	62.50
$CuSO_4.5H_2O$	5	62.50
	10	60.97
	15	35.71
	20	6.25
	25	—

Trace elements	Amount of trace element solutions per 100 ml of Le Mense medium (ml)	Average proteolytic value (PV)
	5	62.50
	10	62.50
	15	62.50
	20	62.50
$Co(NO_3)2.6H_2O$	25	62.50
	30	83.33
	35	83.33
	40	75.72
	45	35.71
	50	35.71
Control	—	62.50

PV of the culture filtrate was markedly improved from 62.50 to 119.30 and 108.69 in the medium supplemented with 20 and 25 ml of $ZnSO_4$. Concentrations of 10 to 20 ml of $CaCl_2$ and 30 ml of $Co(NO_3)_2$ increased the PV from 62.50 to 83.33. An improvement in enzyme formation was also noted with the use of 10 ml of silico-molybdenum complex with a resulting PV of 80.64. A slight increase of 75.72 was obtained with the use of 20 ml of $MnCl_2$. No pronounced effect was observed using H_3BO_3 and $CuSO_4$. Increased concentrations of H_3BO_3, $MnCl_2$, $CuSO_4$, and $Co(NO_3)_2$ were inhibitory to protease production as shown in Table 5.

Inclusion in the medium of a mixture of the favorable trace elements did not increase protease production but the same results were obtained when they were used separately.

Davies [11] found out that trace elements such as Ca, Mg, Mn, Fe, Zn, Cu, Co, Mo are needed for growth and are essential for the activity and stability of many extracellular enzymes. Calcium and zinc have also been described as stimulatory to the activity of the proteinases of *B. subtilis*.

Precipitation of protease.—The clear filtrate with a PV of 125 obtained from the harvested culture yielded 1.5 per cent of protease. The PV of the crude enzyme was 1,660. The crude enzyme is neutral to brownish cream in powdered form (Plate 2).

DESCRIPTION OF THE SELECTED SOIL BACTERIUM

Morphological characteristics.—The cells appear as motile rods with rounded ends, occurring either singly, in pairs, or in short chains (Plate 3, fig. 1). Cells from young cultures are Gram positive and stain uniformly. Cells are spore-forming,

ellipsoidal to cylindrical and are located centrally or paracentral (Plate 3, fig. 2). Individual cells measure from 0.6 to 0.8 μ by 1.5 to 3.0 μ.

Cultural characteristics.—Agar colonies—On tryptone-glucose-yeast-agar, growth appears as irregular colonies with flat, dull, and slightly wrinkled surface, undulate margin, creamy white or with brownish tinge, slightly spreading (Plate 4, fig. 1).

On plates of skimmed milk agar, the bacterium produced clear zones around the colony (Plate 4, fig. 2) indicating hydrolysis of casein.

Agar slants—On tryptone-glucose-yeast agar, the growth is abundant, wrinkled, slightly adherent, cream-colored, and becoming a little brownish with age (Plate 5).

Broth—Clear with heavy, wrinkled, tough pellicle (Plate 6).

Physiological characteristics: Milk: Curd formation at neutral reaction to slightly alkaline, slowly peptonized.

Acid but no gas from glucose, fructose, mannose, maltose, sucrose, glycerol, and mannitol. No acid produced from lactose.

Starch was hydrolyzed.

Acetylmethylcarbinol produced.

Citrates utilized.

Nitrites produced from nitrates. No gas produced from nitrate broth.

Aerobic

Temperature relations: Optimum growth temperature ranged between 28° and 40°C. The maximum temperature for growth is 45°C.

Accessory growth factors such as amino acids are not essential.

IDENTITY

By comparing the description of the genus *Bacillus* which was described by Breed *et al* [12] with that of the bacterium under study and the identified *Bacillus subtilis*, the authors are convinced that the isolate belongs to the *Bacillus* group of the family Bacillaceæ. The morphological, cultural, and physiological characteristics of the identified *B. subtilis* and the soil bacterium are strikingly similar, hence the organism under investigation will be referred to as a strain of *Bacillus subtilis*.

SUMMARY

Nine hundred-ninety bacterial isolates derived from soil were tested in the preliminary screening for their protease-producing efficiency. Eight hundred-sixty were selected based on their ability to produce clear zones on casein agar.

Seventeen of the isolates were capable of producing a PV of 50.0 to 83.33 (Oshima and Church value) in soybean medium. After repeated culturing, B-832 was selected as the most efficient protease producer.

Optimum pH for growth and production of protease is 6.0 to 7.5.

Trace elements, Zn and Ca are stimulatory to protease formation.

The most suitable concentration of ground soybeans on culture medium for enzyme production was 10 to 15 per cent.

Duration of incubation for protease production was 4 days.

Best age of inoculum for enzyme production was 48 hours.

A yield of 1.5 per cent protease with a PV of 1,660 was obtained from the culture medium containing 10 per cent soybeans.

The selected isolate whose morphological, cultural and physiological characteristics are described, was identified as a strain of *Bacillus subtilis*.

ACKNOWLEDGMENT

The authors are indebted to Dr. Clara Lim-Sylianco, professor, University of the Philippines and consultant on microbial enzymes, Biological Research Center, National Institute of Science and Technology, for technical guidance in the course of the work; to Mrs. Luz Baens-Arcega, former director, Biological Research Center, National Institute of Science and Technology, for suggesting the subject of the research and for reviewing the manuscript; to Dr. Patrocinio S. Santos, director, Biological Research Center, National Institute of Science and Technology, for encouragement in the work; and to professor Romeo V. Alicbusan, supervising scientist, Biological Research Center, National Institute of Science and Technology, for reading the paper.

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ILLUSTRATIONS

PLATE 1

Showing 4-day-old culture of the proteolytic bacterium in Le Mense medium containing 10 per cent soybeans.

PLATE 2

Showing the crude enzyme in powdered form.

PLATE 3

FIG. 1. The soil bacterium (*Bacillus subtilis*) from a 14-hour-old culture on tryptone-glucose-yeast-agar medium, stained with crystal violet, to show the rods with rounded ends.

2. Spore-forming cells, from 18-hour-old culture on tryptone-glucose-yeast-agar medium, stained with crystal violet. × 1,250

PLATE 4

FIG. 1. Showing colonies of the soil bacterium from 24-hour-old culture on tryptone-glucose-yeast-agar medium.

2. Showing a colony of the bacterium from 24-hour-old culture on casein agar surrounded with a clear halo as a result of the hydrolysis of casein by the isolate.

PLATE 5

On tryptone-glucose-yeast-agar medium showing the abundant and wrinkled growth of the bacterium.

PLATE 6

Showing the pellicular growth of the bacterium on tryptone-glucose-yeast broth.

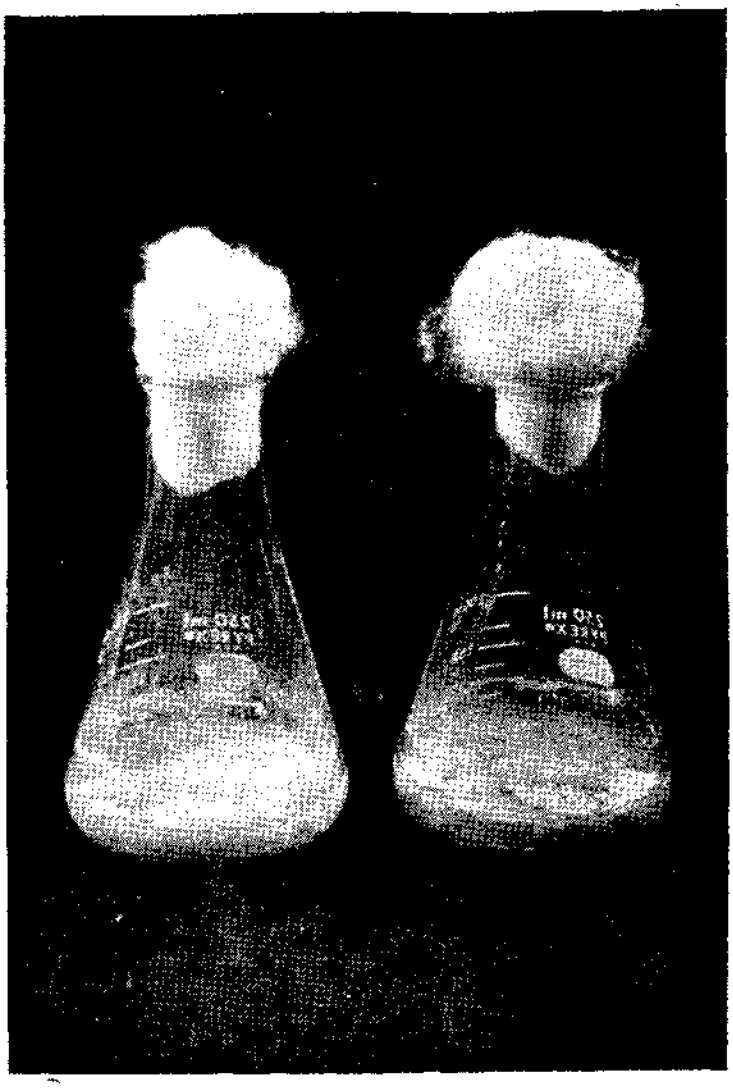


PLATE 1.

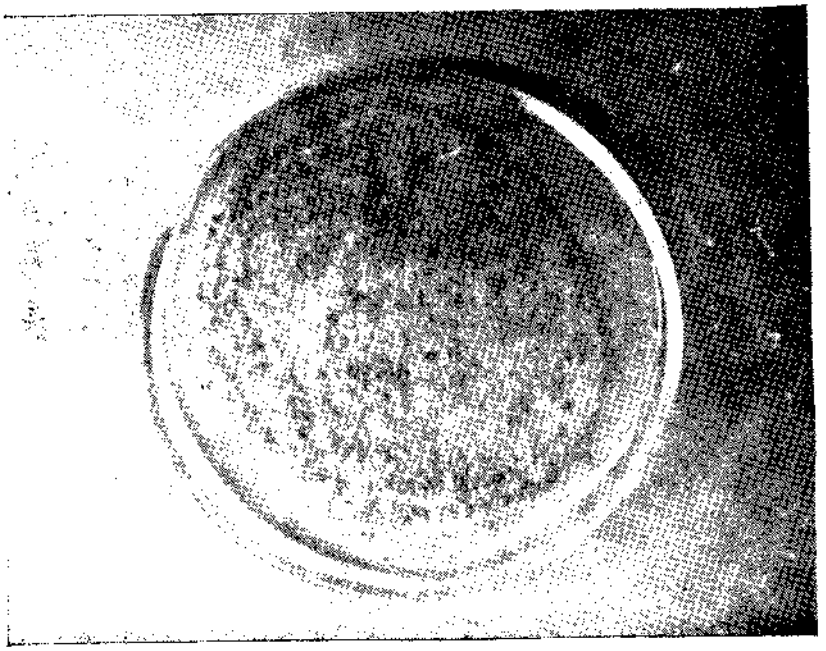
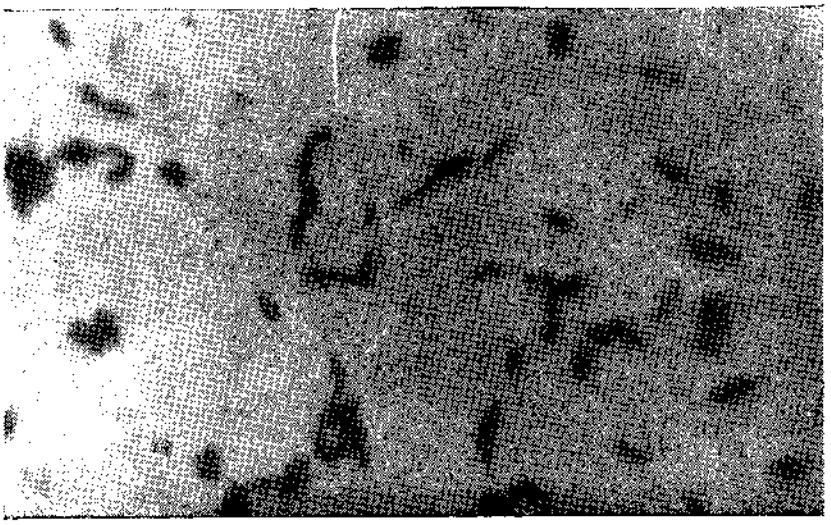


PLATE 2.



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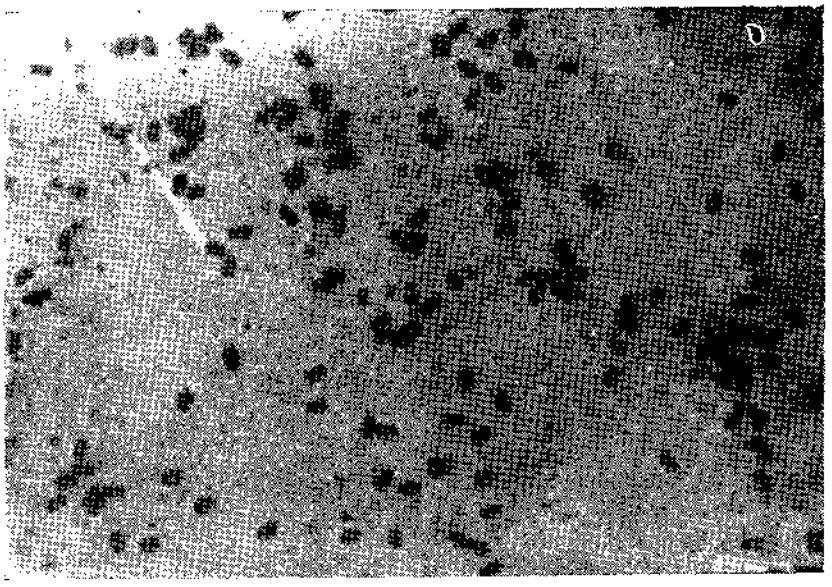

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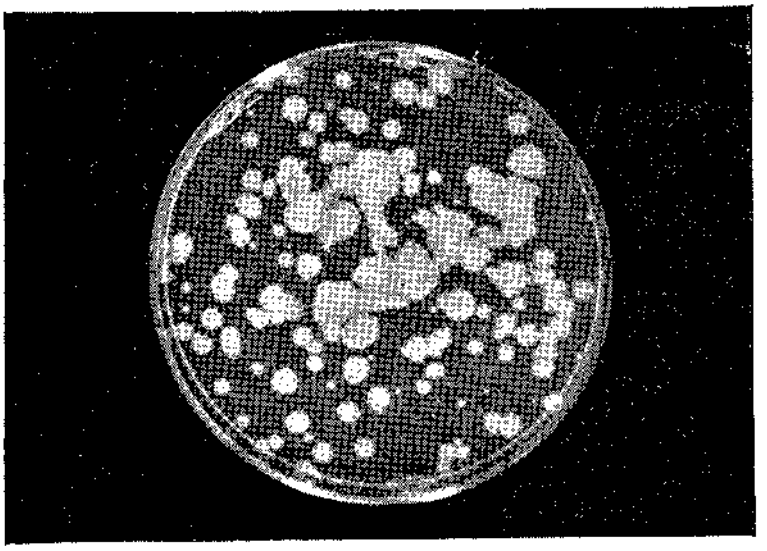
PLATE 3.



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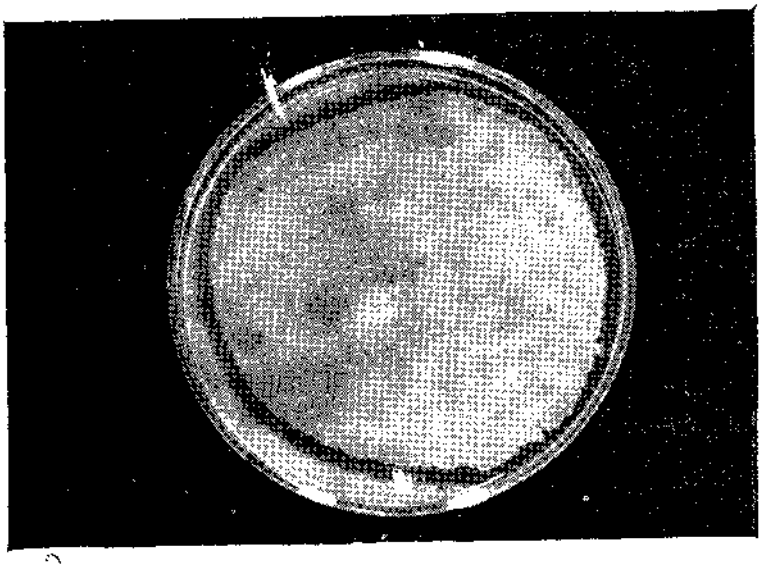

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PLATE 4.

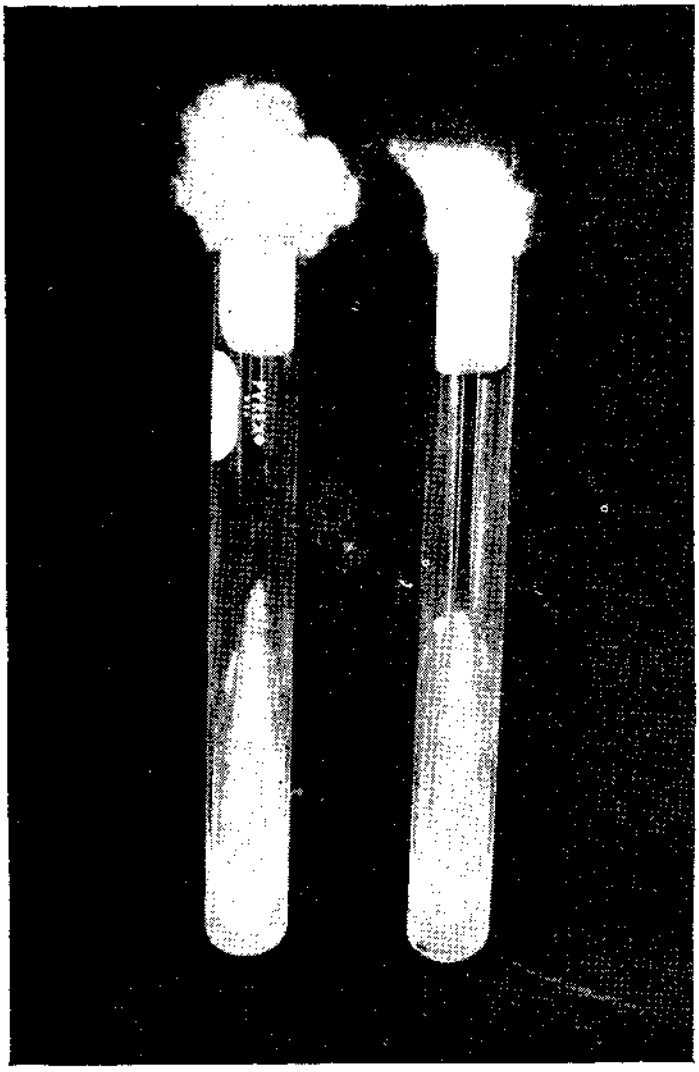

PLATE 5.

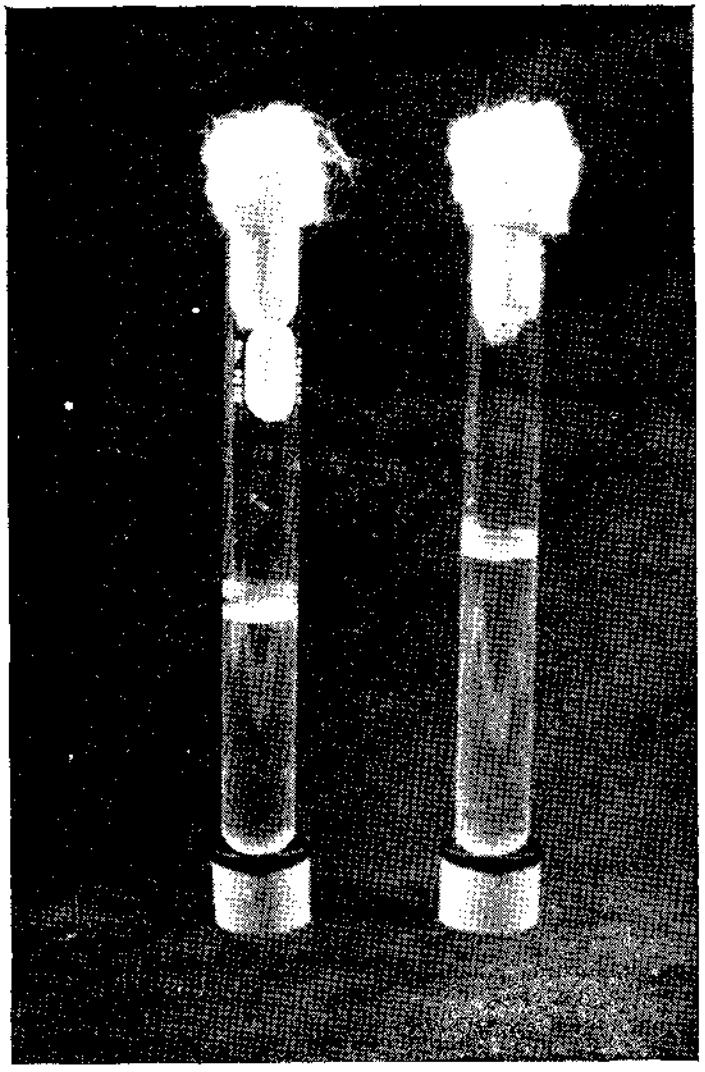

PLATE 6.

MORPHOLOGICAL RESPONSE OF RICE SEEDLINGS TO DINITROXYLIDINE HERBICIDES *

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ABSTRACT

The herbicides, N-(1-ethylpropyl)-2, 6-dinitro-3, 4-xylidine (AC-92553) and N-sec-butyl-2, 6-dinitro-3, 4-xylidine (AC-92390) were tested for their morphological effects on rice at sublethal concentrations. No adverse effect on the germination of rice was obtained with both herbicides. However, the elongation of the shoot and root was inhibited accompanied by swelling of one or both apical meristems. Abnormal enlargement of the cells was apparent in all swollen tissues except at the area of the apical initials.

INTRODUCTION

Dinitroxylidines represent a new group of selective herbicides that have shown promise for use in weed control in transplanted rice. They induce root growth inhibition particularly of grasses and less so in broadleaf weeds. They are structurally related to the nitroanilines, such as trifluralin, which are also herbicidally active and which possess a similar spectrum of weed control. In this study, the morphological response of rice to N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine (AC-92553) and N-sec-butyl-2,6-dinitro-34-xylidine (AC+92390) was investigated to gain an insight into the phytotoxic action of this new group of herbicides.

MATERIALS AND METHODS

One set of rice seeds cv. C_4-63G was germinated in Petri dishes each containing 10 ml of 0, 5, 10, 25 or 50 ppm AC-92390 or AC-92553. Another set was germinated in water on

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enamel trays, and at desired stages, 20 uniform seedlings were transferred to Petri dishes containing 0, 5, 10, 25 or 50 ppm AC–92390 or AC–92553. Each treatment in both sets was replicated three times, each replicate containing 20 seeds or seedlings. Both sets were kept in the dark and observations were made on the elongation of the shoot and root. Any abnormality was noted.

For histological study, root and shoot apices of seedlings treated with 0 and 50 ppm AC–92553 or AC–92390 were fixed in either Crafs III solution or FAA. The tissues were dehydrated through a graded series of tertiary butyl alcohol and embedded in paraffin. Longitudinal sections, 10 to 12 μ thick were cut and stained following the safranin O–fast green schedule. Near median sections were examined and photomicrographed.

RESULTS AND DISCUSSION

Neither AC–92390 nor AC–92553 up to 50 ppm concentration adversely affected the germination of rice (Table 1). However, the elongation of the root and the shoot was inhibited in the subsequent stages of development (Table 2). AC–92553 seems to be more phytotoxic to rice than AC–92390 as shown by the greater inhibition induced by AC–92553 at 5 ppm. With both herbicides, root growth was more sensitive than shoot growth. However, the same saturation levels were observed between 5 and 10 ppm for both organs in each herbicide.

TABLE 1.—*Germination of rice in different concentrations of AC–92553 and AC–92390.*

Conc. (ppm)	Per cent germination *	
	AC–92553	AC–92390
0	100	85
5	100	90
10	100	95
25	100	100
50	100	85

* Average of three replications, taken 3 days after sowing.

TABLE 2.—*Root growth and shoot growth of rice seedlings in different concentrations of AC–92553 and AC–92390.*

Conc. (ppm)	Length (mm) AC–92553		AC–92390	
	Root	Shoot	Root	Shoot
0	13.7	9.2	14.4	7.9
5	1.9	6.9	6.0	4.6
10	1.2	5.3	4.2	4.6
25	1.9	5.9	3.2	4.7
50	2.3	5.1	3.6	4.6

* Treatments were made on 3-day-old seedlings. Measurements were taken after 24 hours.

In addition to inhibition of the normal elongation process, both herbicides caused very conspicuous swelling of the root tip resulting in a bulbous appearance (Plate 1, figs. 1 and 2). Such manifestations are similar to those induced by trifluralin, a nitroaniline, in rice [1] and corn.[2, 3] Anatomical examination revealed that the swelling in the root apex is caused by abnormal enlargement of the cells in all tissues (Plate 2, figs. 1 and 2). The cell dimensions nearly doubled or slightly more than doubled. The epidermal tissues was disrupted and the cortical tissue was less organized.

An added effect of AC–92390, which was not observed in rice seedlings treated with AC–92553, was the inhibited formation of lateral roots (Plate 3, fig. 1). However, when the treatment was prolonged lateral roots were formed at the tip, where swelling was conspicuous, in seedlings treated with 25 and 50 ppm (Plate 3, fig. 2). These manifestations were apparent in trifluralin-treated rice seedlings only when contact with the herbicide was discontinued or when treated with phenylalanine or aspartic acid.[4] As shown in Plate 4, fig. 1, the lateral roots formed at the bulbous tip appear to be of pericyclic origin. Since lateral roots are normally formed a few centimeters away from the tip, the formation of lateral roots at the tip indicates that the pericyclic cells at this region have differentiated to that stage in which they are physiologically capable of giving rise to lateral roots. At 5 and 10 ppm where swelling of the root tip is not apparent, lateral roots were formed at the same region as that in the control.

AC–92390 also caused swelling of the shoot whereas AC–92553 did not give such effect. Anatomical examination of the shoot from AC–92390-treated seedling showed enlarged cells of all tissues except the "shoot apex proper," the area above the youngest leaf primordium (Plate 5, figs. 1 and 2). The cells appeared more vacuolate and less organized. There was, however, no multinuclearity observed as in trifluralin-treated seedlings. The absence of enlarged cells in the AC–92553-treated seedlings may indicate a slower mobility of the herbicide although absorption through the root may take place just as rapidly.

The cells of the developing leaf primordium are in the state of cell division similar to that in the shoot apex proper. However, only the leaf primordium is affected by the herbicide. In the root apex, abnormal expansion of the cells is not evident at the very tip where the initials are located. Enlargement began three or four layers away from the tip. This seeming immunity of the shoot apex proper and the root tip to sublethal concentrations of nitroxylidine herbicides tested suggests a prerequisite for a specific physiological state of the cell in order that it may respond.

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ILLUSTRATIONS

PLATE 1

FIG. 1. Rice seedlings treated with AC–92390 showing inhibited growth of the shoot and root and the bulbous root.

2. Rice seedlings treated with AC–92553 showing inhibited growth of shoot and root and the bulbous root.

PLATE 2

FIG. 1. Root tip from an untreated rice seedling, 40 ×.

2. Root tips taken from rice seedlings treated with 25 ppm AC–92553 and AC–92390, 40 ×.

PLATE 3

FIG. 1. Three-day-old rice seedlings treated with different concentrations of AC–92390. Note the inhibited lateral root formation. Photographed 24 hours after treatment.

2. Three-day-old rice seedlings treated with different concentrations of AC–92390 for 4 days. Note the formation of lateral roots at the swollen root tip at 25 and 50 ppm.

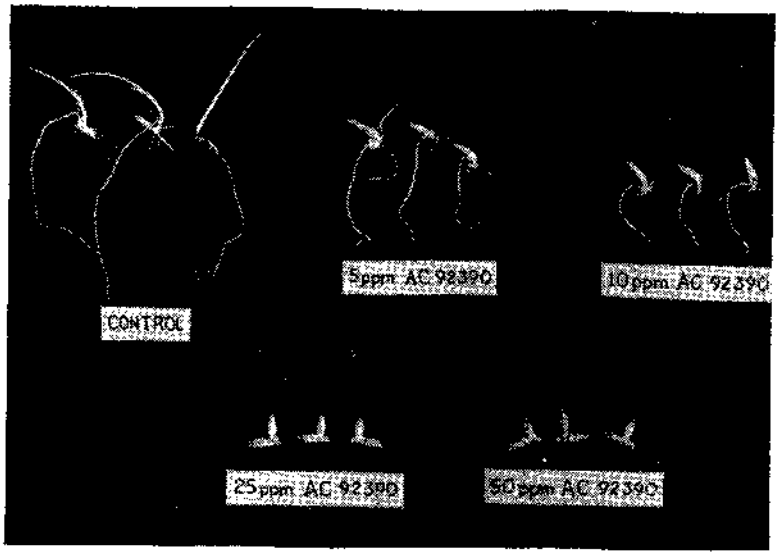
PLATE 4

FIG. 1. Root tip taken from 5-day-old rice seedlings treated with 25 ppm AC–92390 showing the lateral root primordia at the swollen area.

PLATE 5

FIG. 1. Shoot apex from an untreated rice seedling. 100 ×. *sa*= shoot apex proper; *lf*=leaf primordium.

2. Shoot apex from a rice seedling treated with AC–92390 100×. *sa*=shoot apex proper; *lf*=leaf primordium.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10ppm AC 92390
25ppm AC 92390
50ppm AC 92390

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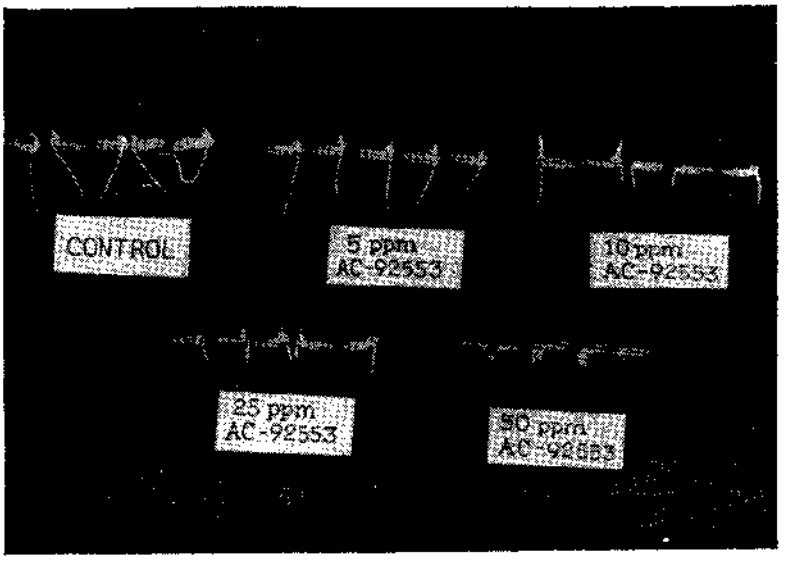
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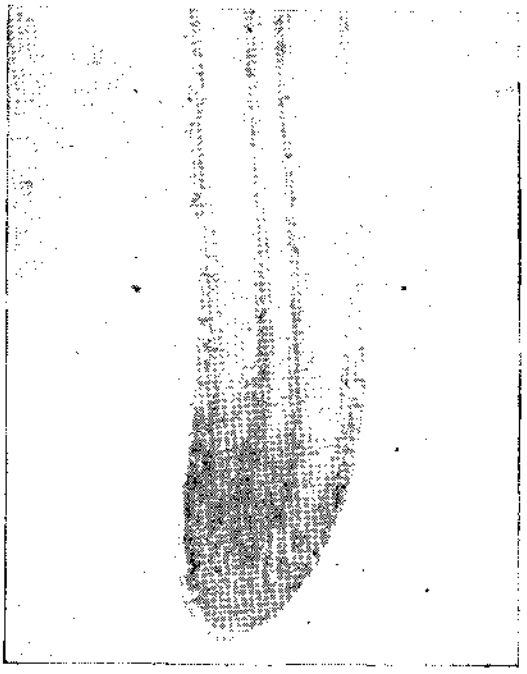
10 ppm AC-92553
25 ppm AC-92553
50 ppm AC-92553

2

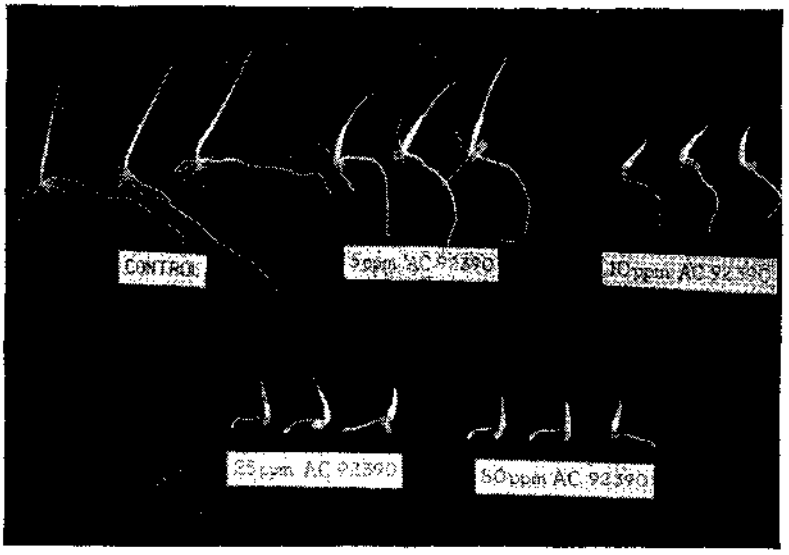
PLATE 1.



1

2

PLATE 2.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10ppm AC 92390
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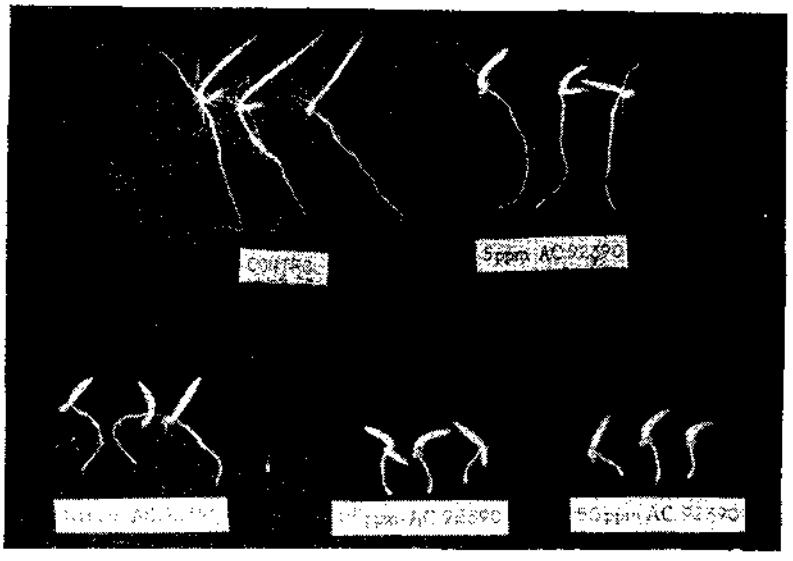
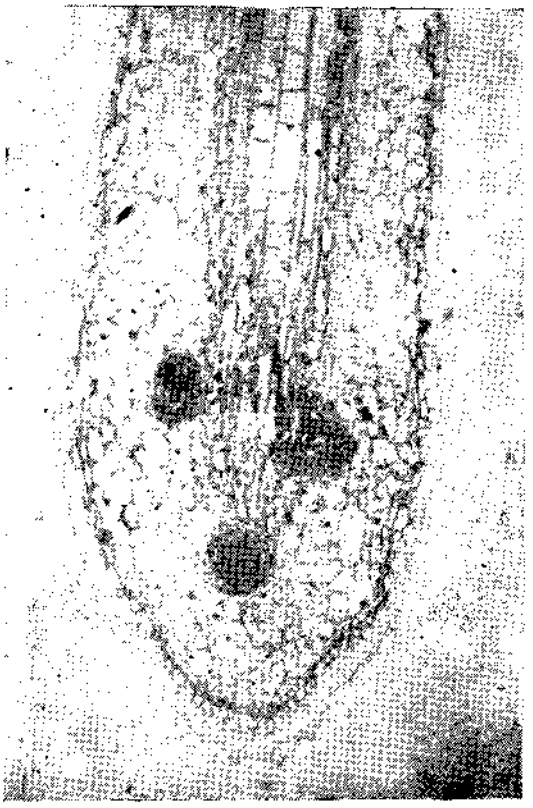
50ppm AC 92390

1


CONTROL
5ppm AC 92390
50ppm AC 92390

2

PLATE 3.



1

PLATE 4.

1

2

PLATE 5.

SHORT COMMUNICATION

TRICYRTIS IMELDÆ, A NEW PHILIPPINE LILY

By HERMES G. GUTIERREZ
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(Received for publication, September 10, 1973.)

In August 1972, an ethnobotanical study was undertaken in Tasaday, South Cotabato. Among the useful plants a new species was discovered, *Tricyrtis Imeldæ*.

The genus *Tricyrtis* of the family Liliaceæ is a new record for the Philippines and the Malaysian region, having been found in Mindanao, over 1,500 kilometers south of its previously known distribution. There are now at least 21 species of this Asian genus known, Japan (12), Taiwan (5), the Himalayas (1), Manchuria (1), Korea (1), and Philippines (1).

TRICYRTIS IMELDÆ H. G. Gutierrez, sp. nov. Fig. 1.

T. stoloniferæ Matsum. tawanensis arcte affinis sed floribus viridi-albus maculis parvis perspicuis purpureis adaxialis ornatis, segmentis externis oblongi-spatulatis apicibus mucronulatis, segmentis internis anguste oblanceolatis præcipue differt.

Perennial herb, *c.* 60 to 70 cm high; rhizomes short, creeping; stem erect, slightly reclinate above, unbranched, puberulous, becoming glabrous with age. Leaves alternate, membranaceous, yellowish-brown above, grayish-brown beneath when dried, the lower ones narrowly elliptic-lanceolate, apex acute to acuminate, base cuneate, sheathing, the upper broadly lanceolate to elliptic, abruptly acute, base more or less cordate, clasping, blade (6-) 12 to 16 cm long, (3-) 4 to 5 cm wide, approximately three times as long as broad, glabrous except on the laxly hairy prominent midrib and main veins beneath, sunken above; main veins three on each side of midrib, seemingly pinnate, converging upwards but not joined at the apex, interconnected by thinner veins and faintly visible reticulate venules. Inflorescence a terminal raceme, furcate, somewhat dichotomous, *c.* 18 to 20 cm long, puberulous, somewhat glaucous. Flowers terminal, greenish-white, bisexual, rather large, showy, with purple spots inside, to over 3 cm long, infundibuliform, glabrous, the pedicel short, 3 to 5 mm long, puberulous. Tepals 6, 2-seriate, free to base, the segments imbricate or the outer series valvate, the three outer segments (sepals) oblong-obovate to oblong spathulate, mucronulate at the tip,

2-lobately saccate at the base, the three inner segments (petals) linear-oblong to narrowly oblong-oblanceolate, pointed at the tip, slightly oblique at base. Stamens 6, the filaments 16 to 18 mm long, slightly flattened; anthers 2-celled, oblong 3 mm long, yellowish-brown, versatile, extrose, dehiscing by vertical slits. Pistil 1, the ovary superior, oblong, 10 mm long, 2 mm

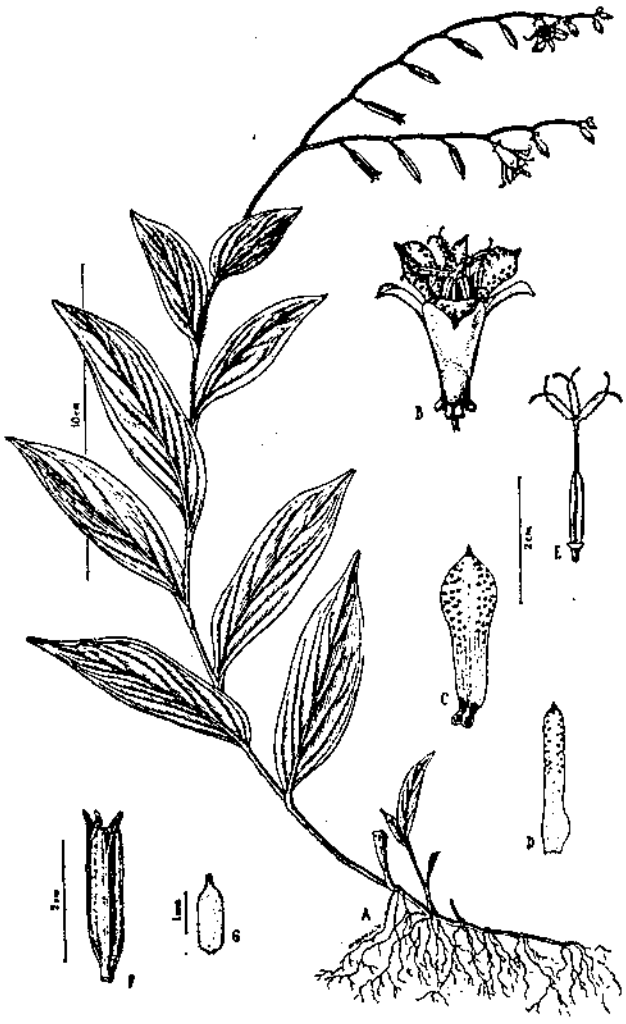

FIG. 1. *Tricyrtis Imeldæ* H. G. Gutierrez: *A*, Habit; *B*, flower; *C*, sepal; *D*, petal; *E*, pistil; *F*, fruit; *G*, seed (from PNH 108822, the type).

wide, 3-angled, trilocular with axile placentation, the ovules numerous, biseriate; style columnar, 8 mm long, as long as the stigmas; stigmas 3, purple, spreading, bifid, tuberculate on the inner side. Fruit a septicidal capsule, narrowly oblong or linear-trigonous, *c.* 25 to 30 mm long, 4 to 6 mm wide; seeds oblong, pointed at the tip, about 2 mm long, flat.

MINDANAO, Tasaday, Cotabato, primary forest, along stream, alt. *c.* 1,300 m, August 2, 1972, *Gutierrez, Reynoso,* and *Yen PNH 108822,* (type, PNH). Observed to be rather rare in the area. Attractive as an ornamental plant.

VERNACULAR NAME: *Amutmagiso,* Tasaday.

The leaves, although quite thick and fleshy when fresh, become thin and membranaceous when dried. The purple spots on the inner side of the perianth likewise disappear in the process of drying.

Among the Tasadays, the flowers or the juice from the leaves of the plant is rubbed on the hands preparatory to frog-catching. This is said to be attractive to the quarry, and renders it unslippery to the hand. To the Tasadays, the perianth segments resemble a frog's tongue. The flowers are also eaten by the natives.

This species is named in honor of Mrs. Imelda Romualdez Marcos in recognition of her continuing contributions to the upliftment of the Philippine cultural minorities including the Tasadays.

ACKNOWLEDGMENT

The author is grateful to John Nance for providing the photographs; to the National Research Council of the Philippines for a grant to enlist the services of Ricardo C. Aguilar for the illustration; to PANAMIN (Presidential Assistance on National Minorities) for the opportunity to join the Tasaday expedition; to Ernesto J. Reynoso, senior technician, National Museum, who assisted in the field; and to Douglas E. Yen, co-researcher in the ethnobotanical study of the Tasaday.

LABORATORY STUDIES ON THE PREPARATION OF SKIM MILK CONCENTRATE

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(Received for publication, October 18, 1973.)

ABSTRACT

A method of preparing concentrated skim milk from the skim milk by-product of the wet processing of coconut meat was developed at the National Institute of Science and Technology. The product was prepared by the addition of sugar to and subsequent concentration of the skim milk to give a reconstitutable acceptable concentrate packed in tin cans. The effect of moisture and sugar levels on the keeping quality of the concentrate was determined. Organoleptic assessment, microbiological, and chemical examinations were used in the evaluation of product acceptability.

INTRODUCTION

The literature [1-11] is replete with researches conducted on the development of food products from coconut. With the wet processing of coconut [12] starting to make a headway in the field of coconut technology, the utilization of the by-products derived from the process offers new and interesting areas for research.

Gonzales *et al* [13] developed a laboratory process of preparing canned coconut cream (*gata*) thus extending the shelf life of what was previously a very unstable emulsion of coconut cream and water to a storage period of more than one year. The process, the essential features of which are described by Gonzales, extracts the oil from the fresh meat by the wet process. Protein solids, wet meal, and skim milk are obtained as by-products of the process. Recent researches are thus directed towards the utilization of these three by-products.

Gonzales *et al* [14] made studies on the preparation of cocohoney using as the principal base of their product the skim milk obtained from the process. A part of the protein solids

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from the de Laval centrifuge was added to the preparation to improve the nutritive quality of the product.

In 1968, Mañalac and co-workers [15] succeeded in preparing a dry, creamy white, flaky products by simply drying the preconcentrated skim milk in a vacuum oven. The preparation which came to be known as Instant Skim Milk was highly hygroscopic, keeping well for a period of more than one year when packed in polyethylene bags or in bottles and stored in a dessicator. Upon reconstitution with ice cold water and the addition of just enough sugar to taste, a delicious, refreshing, *buko*-flavored drink was obtained. The researchers claimed the superiority of the instant skim milk product over the commercial soft drinks in that the former is more nutritious considering the amount of protein in the concentrate. The chemical analysis of the product in its dry and normal reconstituted form is shown in Table 1. Extensive storage studies and acceptability tests could not, however, be conducted on the flake product since it was not possible to prepare enough working samples to cover studies for a longer period of storage. In order to prepare a sizeable amount of

TABLE 1.—*Chemical analysis of instant coco skim milk beverage.*

Product	Water	Oil	Protein
	Per cent	*Per cent*	*Per cent*
Instant coco skim milk	4.45	0.46	25.20
Reconstituted powder	91.08	0.172	1.01

sample, the laboratory vacuum oven, which was originally used for drying the skim milk becomes inapplicable since a thin film of the liquid requires a drying time of at least 15 hours. Rather, a drum dryer provided with appropriate vacuum accessories or a spray dryer must be used for further drying of the product. Furthermore, the hygroscopic nature of the product presents packaging as well as storage problems.

This study was therefore undertaken in order to utilize the skim milk by-product from the production of canned gata by the wet method and to explore the potentials of such by-product as a source of nutritious beverage. In addition, this study seeks to improve on a previous method of preparation of instant coconut skim milk.

EXPERIMENTAL

Materials and methods.—The skim milk used in this study was obtained from the processing of coconut meat by wet method. Coconut meat from matured nuts were passed through the electric grater and the whole coconut milk extracted from the meat by means of the Carver press. Two additional pressings through the Carver press were done with the meal with an amount of water corresponding to one-half the original weight of the coconut meat.

The extracts from the three pressings were pooled together and the total extract passed through the de Laval Centrifuge. The skim milk obtained was then used in the preparation of coco skim milk concentrate.

Refined cane sugar of 99 per cent purity was used for adjusting the sugar level of the skim milk product.

Preparation of coconut skim milk concentrate.—The schematic diagram for the preparation of coco skim milk concentrate is shown in Figure 1.

Separate batches of skim milk of 500 g each was preconcentrated in a laboratory rotary flash evaporator to remove 50 per cent of the original amount of water of the milk. A precalculated amount of sugar was then thoroughly blended with the preconcentrated skim milk and evaporation continued to the desired moisture level.

Six sets of the skim milk concentrate product were prepared with varying moisture and sugar levels; namely, Set I with 15 per cent sugar, 20 per cent water; Set II with 15 per cent sugar, 25 per cent water; Set III with 15 per cent sugar, 30 per cent water; Set IV with 10 per cent sugar and 20 per cent water; Set V with 10 per cent sugar and 25 per cent water; and Set VI with 10 per cent sugar, and 30 per cent water.

The concentrated products were hot-packed in sterile tin cans and stored in well-ventilated shelves.

Examination of samples.—All the six different sets of coconut skim milk concentrate were stored at ambient temperature and the stored samples evaluated at intervals of 2 weeks for a total storage period of 6 months.

Both the freshly prepared products and the stored samples were subjected to the following routine examination of food samples.

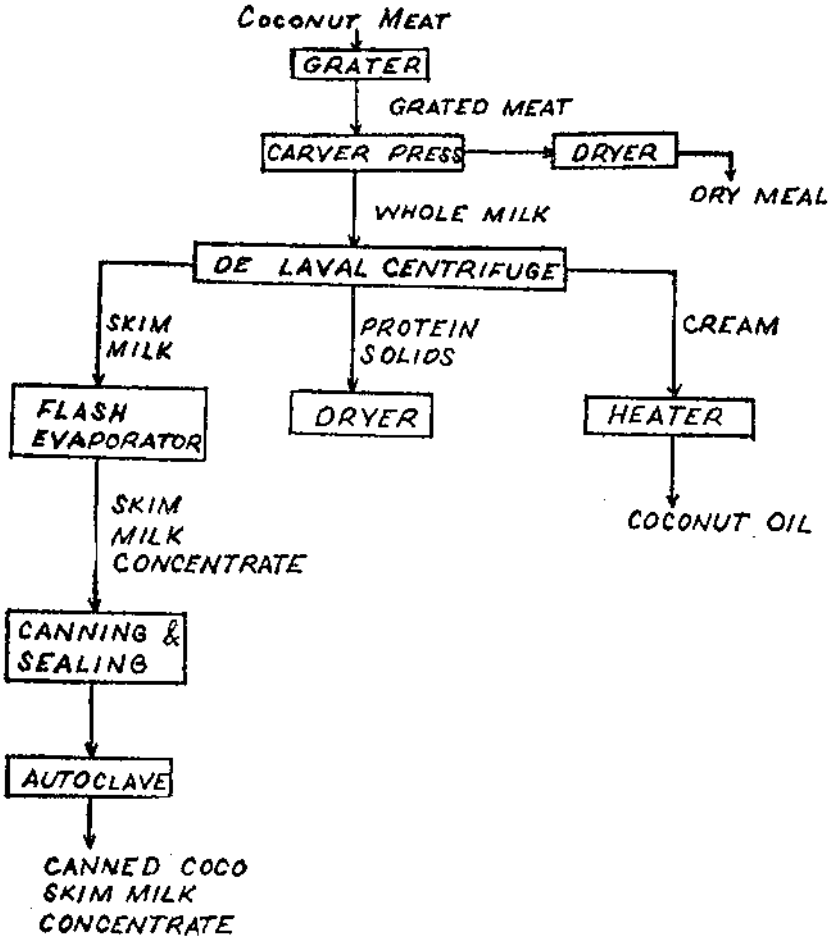

FIG. 1. Laboratory method for the preparation of coconut skim milk concentrate.

Chemical analysis.—The products were analyzed for moisture, oil, protein, total sugars, titratable acidity, and pH.

The moisture and oil contents were determined according to the official methods of analysis of the Association of Official Analytical Chemists (AOAC) while protein content was determined by the micro-Kjeldahl method for nitrogen determination and subsequently mutliplying the percentage nitrogen by the factor 6.25. The Munson and Walker method was used for determining the amount of total sugars while the titratable acidity expressed as per cent $HC_2H_3O_2$ was determined by

titration with standard alkali. The pH values of the samples were measured by the use of the Beckmann Zeromatic pH meter.

Microbiological examination.—The total microbial population of both the fresh and stored samples were determined using the agar plate count method.

Organoleptic assessment.—A panel consisting of six experienced members from the FNRC, NIST, selected for their taste acuity and consistency, made a sensory evaluation of the product. A score sheet on preference tests of food samples prepared by the Technical Committee of the FNRC and approved by the Office of Statistical Coordination and Standards, National Economic Development Authority was used. It consists of descriptive terms with corresponding numerical scores as follows: desirable, 10–9; acceptable, 8–7; neutral (neither like nor dislike), 6–5; objectionable, 4–3; unacceptable, 2–1.

RESULTS AND DISCUSSIONS

The data in Table 2 show that all the six sets of freshly prepared coco skim milk concentrate were given acceptable organoleptic scores by the panel of tasters. Similarly, all the fresh samples had innumerable microbial colonies indicating that aseptic care was not taken in the handling and preparation of the samples.

The analysis of variance (ANOVA) table for the effect of moisture content and storage period on the acidity of the different sets of coco skim milk concentrate is shown in Tables 3 and 4. Statistical evaluation indicates that the two factors; namely, moisture content and storage period have no significant effect on the acidity of samples containing 15 per cent sugar with varying moisture levels. Storage period, however, was found to have slightly significant effect on the acidity of the samples containing 10 per cent sugar with varying moisture levels. These results indicate that the sugar level and not the moisture level of the samples is the vital factor in preventing the onset of microbial fermentation. Thus, samples containing 10 per cent sugar are less resistant to acidity changes on storage than the coconut skim milk concentrate containing 15 per cent sugar. The addition of sugar as a method of food preservation depends upon the reduction of available moisture to a level where the development of microorganisms is prevented. Thus,

TABLE 2.—*Results of organoleptic, microbiological and chemical examination of freshly prepared coconut skim milk concentrate*

Sample	Bacterial count	Preference scores	Chemical analysis							
			Water	Oil		Protein		Acidity		Sugar
				As received	H_2O – free	As received	H_2O– free	Oil H_2O– free	As $HC_2H_3O_2$	
			Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Set I 15 Per cent sugar and 20 Per cent water	Innumerable	8	16.21	0.544	0.65	9.51	11.35	11.54	0.680	58.81
Set II 15 Per cent sugar and 25 Per cent water	Innumerable	8	22.37	1.06	1.36	9.91	12.76	12.94	0.529	46.06
Set III 15 Per cent sugar and 30 Per cent water	Innumerable	8	32.65	0.801	1.19	--	—	—	0.55	44.36
Set IV 10 Per cent sugar and 20 Per cent water	Innumerable	8	22.55	0.983	1.27	12.64	16.32	16.53	0.669	45.81
Set V 10 Per cent sugar and 25 Per cent water	Innumerable	7	29.24	0.693	0.979	—	—	—	0.55	44.93
Set VI 10 Per cent sugar and 30 Per cent water	Innumerable	7	26.96	0.263	0.360	5.40	7.39	7.42	0.351	39.90

TABLE 3.—*Analysis of variance for the effect of moisture content and storage period on acidity of coco skim milk concentrate.*

Sources of variation	Degrees of freedom	Sum of squares	MSS	F-Value (Calculated)	F-Value (from table)
Moisture content	3—1 = 2	0.157	0.0785	*2.9	5.14
Storage period	4—1 = 3	0.2315	0.0771	*2.9	4.76
Errors	6	0.1585	0.0264		
TOTALS		0.547			

* Insignificant at 5 per cent level.

TABLE 4.—*Analysis of variance for the effect of moisture content and storage period on acidity of coco skim milk concentrate: Sets IV—VI.*

Sources of Variation	Degrees of freedom	Sum of squares	MSS	F-Value (Calculated)	F-Value (from table)
Moisture content	2	0.130	0.065	2.7	5.14
Storage period	3	0.559	0.168	7.0*	4.76
Errors	6	0.145	0.021		
TOTALS		0.834			

*Significant at 5 per cent level.

in skim milk concentrate, a concentration of 15 per cent sugar is enough to reduce the water activity to an inhibitory level.

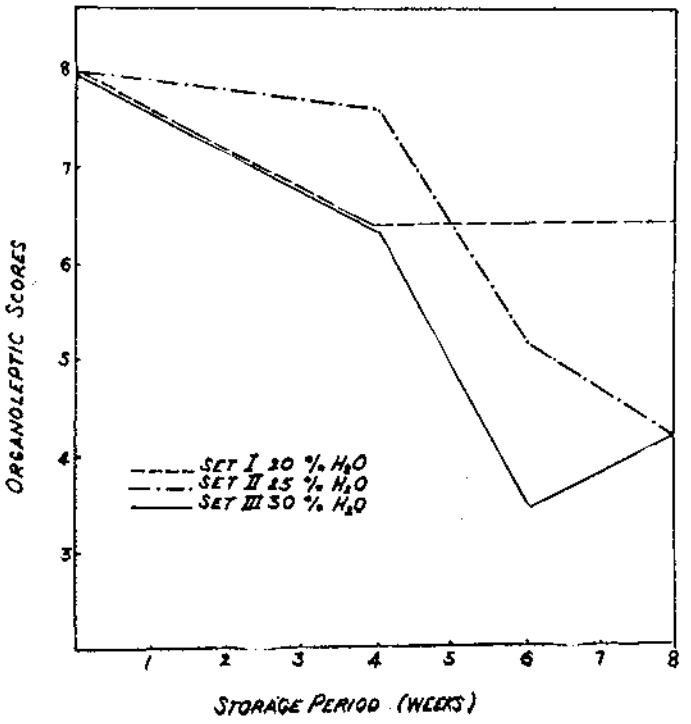

FIG. 2. Organoleptic scores vs. storage period for coco-skim milk concentrate samples at different moisture levels and 15 per cent sugar level.

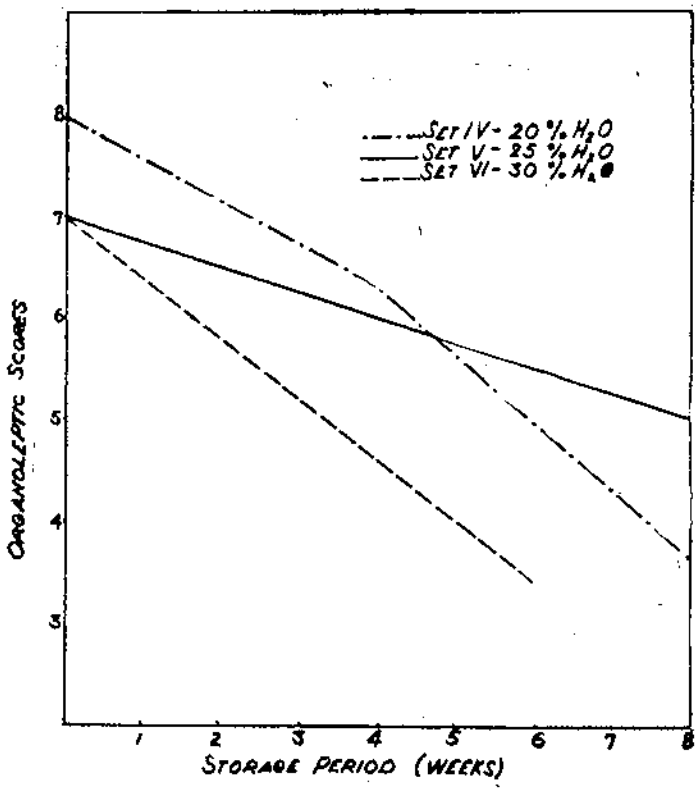

FIG. 3.. Organoleptic scores vs. storage period for coco-skim milk concentrate samples at different moisture levels and 10 per cent sugar level.

Organoleptic scores of the stored samples when plotted against storage time give curves shown in Figures 2 and 3 indicating that although all the samples were rated by the panel as acceptable, when freshly prepared; organoleptic scores for all the samples except set I start to decline steadily upon storage. Set I which contains 15 per cent sugar and 20 per cent water was given a favorable score by the panel even after eight weeks of storage.

The results of the microbiological examination of the stored samples are shown graphically in Figures 4 and 5. From the graphs, it may be observed that microbial count of all the samples except set II remained innumerable after 10 weeks of storage. The microbial population, however, was found to

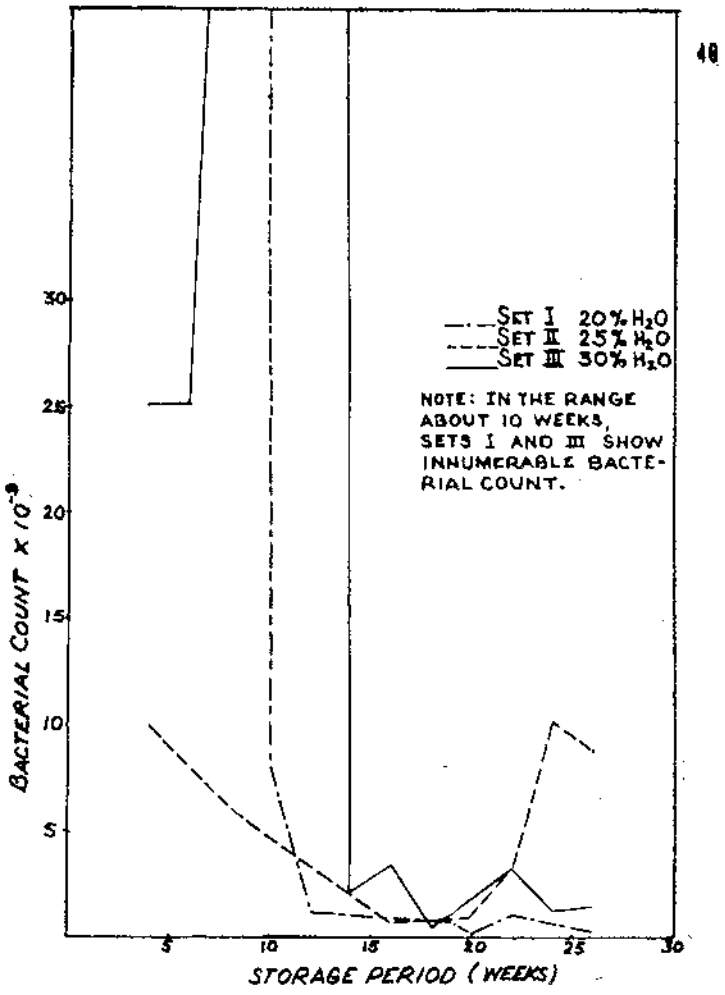

FIG. 4. Bacterial count vs. storage period for coco-skim milk concentrate samples at different moisture levels and 15 per cent sugar level.

decrease rapidly with storage. The curve representing sample set IV is particularly interesting since at the 15th week of storage, microbial count tended to level off at a very low minimum, approaching zero at the end of the storage study.

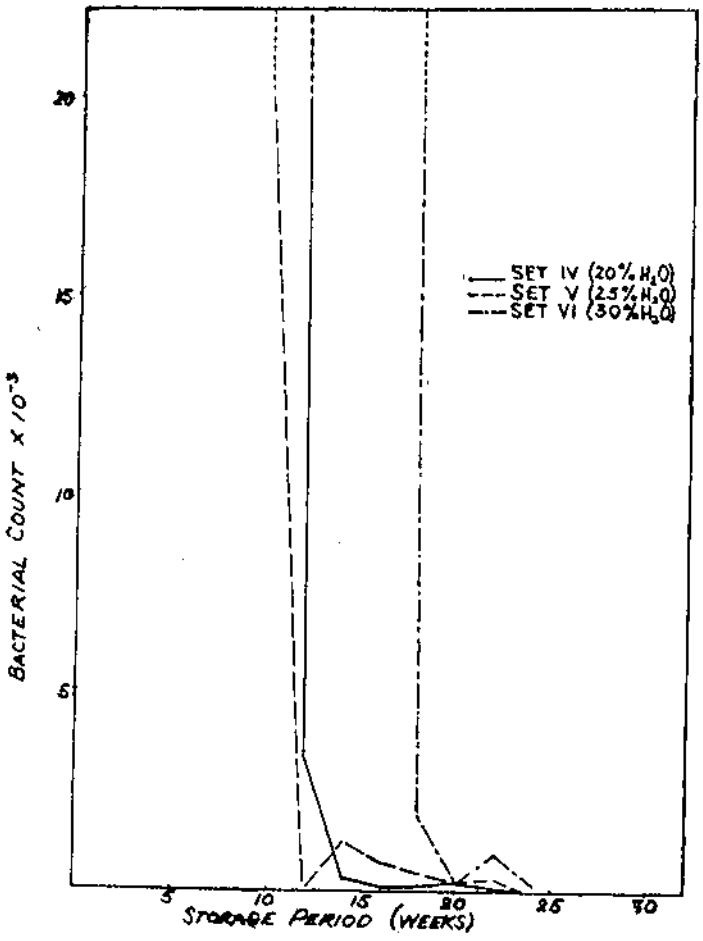

FIG. 5. Bacterial count vs. storage period for coco-skim milk concentrate samples at different moisture levels and 10 per cent sugar level.

Results of both subjective and objective evaluations of the product indicate that moisture and sugar levels of 20 per cent and 15 per cent respectively give an acceptable coco skim milk concentrate. The product is creamy white in color, has a very viscous consistency and can be diluted with ice cold water to twice its volume giving a sweetened coco drink.

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to the corresponding carbonyl derivative and sulfate ions.

$$RNH.CS.NHR' + 4PhI(OAc)_2 + 5H_2O$$
$$RNH.CO.NHR' + H_2SO_4 + 4PhI + 8HOAc$$

where, (*i*) R and R' = H; (*ii*) R = alkyl or aryl and R' = H; (*iii*) R = NH_2 and R' = H.

The double bond in the allyl chain (in allylthiourea) is not attacked.

MATERIALS AND METHODS

Reagents.—Phenyliodosoacetate, 0.05 M, in glacial acetic acid was prepared by the method of Pausacker,[2] and standardized iodometrically. Most of the mercaptans were gifts from Evans Chemetics, New York. Some samples were prepared by established methods. Thiourea solutions were determined by alkaline iodine,[3] and thiosemicarbazide solutions by nitrite.[4] Samples of p-tolylthiourea and allylthiourea were of the maximum purity available.

Procedures.—(*i*) For mercaptans. Weigh the sample containing 0.2 to 3.0 meq of mercaptan into a 150-ml Erlenmeyer flask containing 30 ml of distilled water (through which nitrogen has been bubbled for a long period). If the sample is insoluble in water, dissolve it in 10 to 15 ml of glacial acetic acid and add sufficient water to bring acid concentration to roughly 30 to 40 per cent by volume at the time of titration. Less water may be used if the sample does not give a sharp end point (at high acetic acid concentrations, reactions are slow and the end point is not of the sharpest type). Add 50 mg of potassium iodide and 1 ml of 1-per cent starch solution and titrate with 0.05-M phenyliodosoacetate taken in a 10-ml buret, to the blue color of starch.

(*ii*) For thiocarbonyl substances. Pipet an aliquot of sample, dissolved in water or glacial acetic acid, containing 0.01 to 0.1 mole of thiocarbonyl compound into a 250-ml Erlenmeyer flask. Add glacial acetic acid solution of 0.05-M phenyliodosoacetate, providing for about 50-per cent excess. Prior to the addition of iodosoacetate, add calculated volumes of water so as to keep acid concentration between 30 to 80 per cent by volume. Set aside for at least 30 minutes. Add a measured excess of 0.05-M ascorbic acid solution and back titrate with 0.05-M iodide or phenyliodosoacetate in the usual manner. Do a blank.

Results.—Results given in Table 1 for the determination of several mercaptans show that the method is sufficiently ac-

TABLE 1.—*Determination of mercaptans.*

Mercaptan titrated	Present method* + Average deviation			Other methods (Reference)
2—Mercaptopropionic acid	96.6	0.3	96.4	Iodometry [6]
Mercaptoacetic acid	80.3	0.3	80.0	——do——
2—Diethylaminoethanethiol HCL	98.2	0.2	98.1	——do——
Toluene-α-thiol	98.2	0.4	98.5	Acetylation [7]
1—Pentanethiol	88.0	0.5	88.2	Iodometry
2—Mercaptoethylammonium chloride	97.5	0.3	97.6	——do——
1—Butanethiol	90.6	0.4	90.3	——do——
Allylthiol	93.2	0.2	93.0	Acetylation
s—Butanethiol	97.0	0.3	97.1	Iodometry
Thiobenzoic acid	95.3	0.5	95.0	——do——
p—Chlorotoluene-α-thiol	98.6	0.2	98.9	——do——
Methyl-3-mercaptopropionate	96.4	0.3	96.2	——do——
2—Mercaptoethanol	99.5	0.2	99.3	——do——
Benzenethiol	98.7	0.1	99.0	——do——

* Three or more determinations.

curate. Results for the determination of mercaptans in presence of interfering substances are given in Table 2. The

TABLE 2.—*Interferences.*

Mercaptan titrated	Substance added	Molar ratio added compd=RSH/	RSH* per cent recovery
2—Mercaptopropionic acid	Dextrose	172:1	100.2
	Acrylonitrile	95:1	99.9
	Propyleneglycol	36:1	100.6
	Methylacrylate	10:1	101.0
2—Mercaptoethylammonium chloride	Carbondisulfide	106:1	100.2
	Glycine	83:1	100.0
2—Mercaptoethanol	Formic acid	997:1	100.7
	Dibenzoldisulfide	72:1	100.0
	Sulfasalicylic acid	9:1	99.8
	Thiophene	10:1	100.5
2—Diethylaminoethanethiol HCL	Acetone	245:1	100.0
	Potassium cyanide	83:1	100.7
	Alanine	70:1	100.2
Mercaptoacetic acid	Dimet. ylsulfoxide	314:1	100.0
	Diethylsulfide	14:1	100.0

* Av. of two determinations, per cent recovery taken into account previously determined purity of sample.

interferences studied include compounds that interfere in other methods and compounds that contain other sulfur functional groups. Thiocarbonyl compounds interfere. Quantitative results for the determination of thiocarbonyl compounds are given in Table 3. The procedure is precise to about ± 0.5 per cent. Compounds that react with iodosoacetate interfere.

PUBLICATIONS AVAILABLE

CHECKLIST OF THE ANTS (HYMENOPTERA: FORMICIDAE) OF ASIA. By J. W. Chapman and S. R. Capco. Institute of Science and Technology Monograph 1 (1951) new series. Paper, 372 pages. Price, $2.00, United States currency.

NOTES ON PHILIPPINE MOSQUITOES, XVI. GENUS TRIPTEROIDES. By F. E. Baisas and Adela Ubaldo—Pagayon. Institute of Science and Technology Monograph 2 (1952) new series. Paper, 198 pages with 23 plates and four text figures. Price $2.50, United States currency.

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FERN FLORA OF THE PHILIPPINES. By Edwin B. Copeland. Institute of Science and Technology Monograph 6, Vols. 1–3 (1958–1960) new series. Vol. 1, 191 p., Paper, Price, $1.25; Vol. 2, 193–376 p., Paper, Price, $1.75; Vol. 3, 377–577 p., Paper, Price $1.75, United States currency.

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THE ARCHAEOLOGY OF CENTRAL PHILIPPINES. By Wilhelm G. Solheim, II. National Institute of Science and Technology Monograph 10 (1964) new series. Paper, 235 pages with 29 text figures and 50 plates. Price, $3.00, United States currency.

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